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MOLECULAR AND THERAPEUTIC ASPECTS OF BREAST CANCER STEM CELLS

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Molecular and Therapeutic Aspects of Breast Cancer Stem Cells

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ABSTRACT

Breast cancer stem cells (BCSCs) are defined as a minor cellular component in the solid tumor, displaying mammary stem cell properties by expressing embryonic stem cell genes. They are usually undergoing self-renewal process, but highly potent to give rise to other types of tumor cells and create a hierarchically arranged tumor under certain stimuli. Accumulating evidences suggest that BCSCs are more resistant to conventional therapies and play an important role in mediating tumor metastasis and relapse. Therefore more targeted therapy on the scope of BCSCs is required to improve prognosis of breast cancer patients.

Two estrogen receptors (ER) exist in breast cancer: ERα and ERβ. Around 75% of all primary breast tumors can be identified as ERα positive. In study I, we confirm BCSCs derived from human tumors as well as cell lines are absent of ERα expression but dominant in ERβ expression. We show that ERβ is correlating with phenotypic cancer stem cell markers and responsible for the proliferative role of estrogens and thereby essential for tumor growth. We also observe ERβ activated gene signature in BCSCs associated with poor outcome in three independent breast cancer cohorts. By *in vivo* xenograft experiments we show that agonizing or antagonizing ERβ enhances or inhibits tumor growth respectively. Hence, we suggest that ERβ is a central mediator of estrogen action in BCSCs.

Although BCSCs are considered the apex of hierarchy in breast cancer progression, the origin of this unique cell subpopulation is not well established. Two alternative and contradictive models have been proposed to describe the tumor growth governed by BCSCs. In study II, we observe a large degree of shared mutations as well as similar spread of allele frequencies between BCSCs and the differentiated tumor cells by performing whole exome sequencing. We thereby conclude the existence of BCSCs as a population of cells dynamically and reversibly switching from differentiated tumor cells.

In paper I, we observed that tamoxifen appears to be insufficient to reduce mammosphere formation and proliferation even from BCSCs derived from ERα+ tumors. Therefore, in study III we conducted genome-wide transcriptional analysis to explore potential mechanism posed by tamoxifen. Within the tamoxifen-induced transcriptional pathways, ribosomal biogenesis and mRNA translation are the most

significantly and physiologically relevant pathways. We also identified induction of the key mTOR downstream targets S6K1, S6RP and 4EBP1 in BCSCs by tamoxifen on protein level. Using mTOR inhibitors along or in combination with tamoxifen, we observed significant reduction in mammosphere formation. We conclude that tamoxifen can activate mTOR-signaling pathways in BCSCs associated with endocrine resistance.

The rapid evolving genomic technologies and the advantages of using *ex vivo* modeling widens the possibilities to perform improved diagnostics for precision oncology. In study IV, we report a simple but robust superficial scraping method to collect viable primary cells from small tumors. Validation of scraping material has proven the method to be sufficient for analayses at DNA and mRNA level. By using this method for routine biobanking, the inclusion of resected tumors for fresh frozen sample storage could increase significantly from 60% up to 85% of the total breast cancers.

In conclusion, studying BCSCs as a model system and as a target will be important for development of cancer therapies in advanced breast cancer.

LIST OF SCIENTIFIC PAPERS

I. **Estrogen receptor β governs proliferation of breast cancer stem cells and can be targeted by endocrine therapy.**

RAN MA, G M KARTHIK, FELIX HAGLUND, JOHN LÖVROT, GUSTAF ROSIN, ANNE KATCHY, CAMILLA HILLIGES, LENNART BLOMQVIST, JAN FRISELL, CECILIA WILLIAMS, IRMA FREDRIKSSON, JONAS BERGH, JOHAN HARTMAN

Manuscript

II. **Sequencing of breast cancer stem cell populations indicates a dynamic conversion between differentiation states *in vivo.***

DANIEL KLEVEBRING[1], GUSTAF ROSIN[1], RAN MA, JOHAN LINDBERG, KAMILA CZENE, JUHA KERE, IRMA FREDRIKSSON, JONAS BERGH, JOHAN HARTMAN

Breast Cancer Research. 2014 Jul 6;16(4):R72

III. **mTOR inhibitors counteract tamoxifen-induced activation of breast cancer stem cells.**

G M KARTHIK[1], RAN MA[1], JOHN LÖVROT, LORAND KIS, CLAES LINDH, LENNART BLOMQUIST, IRMA FREDRIKSSON, JONAS BERGH, JOHAN HARTMAN

Cancer Letters. 2015, in press

IV. **Superficial scrapings from breast tumors is a source for biobanking and research purposes.**

RAN MA, IRMA FREDRIKSSON, GOVINDASAMY-MURALIDHARAN KARTHIK, GREGORY WINN, EVA DARAI-RAMQVIST, JONAS BERGH, JOHAN HARTMAN

Labotary Investigation. 2014 Jul;94(7):796-805

[1] *Those authors contributed equally.*

LIST OF RELATED PAPERS

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LIST OF ABBREVIATIONS

AF1	Activation function-1
AF2	Activation function-2
ALDH1	Aldehyde dehydrogenase 1
BCSCs	Breast cancer stem cells
CSCs	Cancer stem cells
DPN	Diarylproprionitrile
DBD	DNA binding domain
ERα	Estrogen receptor α
ERβ	Estrogen receptor β
E_2	Estradiol
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transformation
FACS	Fluorescence-activated cell sorting
HER	Human epidermal growth factor receptor
IGFR	Insulin-like growth factor
LBD	Ligand binding domain
mTOR	Mammalian target of rapamycin
MET	Mesenchymal-epithelial transformation
PDX	Patient derived xenografts
PHTPP	4-[2-Phenyl-5,7-*bis*(trifluoromethyl)pyrazolo[1,5-*a*]pyrimidin-3-yl]phenol
PI3K	Phosphatidylinositide 3-kinases
PPT	Propylpyrazoletrisphenol
PTEN	Phosphatase and tensin homolog

1 INTRODUCTION

1.1 BREAST CANCER INTRINSIC SUBTYPES AND HETEROGENEITY

1.1.1 Breast cancer intrinsic subtypes

Breast cancer is a highly heterogeneous disease showing high degree of diversity among cancer patients and tumors. Traditionally, breast cancer heterogeneity has been observed by histopathological examination on the morphological level, and it is thought to affect clinical outcomes [1,2]. Recently, due to developments in gene expression technologies, the traditional classification methods based on immunohistochemistry and morphology have been refined, and are gradually being replaced by intrinsic molecular classification [3,4]. Breast cancer is one of the few tumor types that can be classified into distinctive molecular subtypes by microarray-based gene expression analysis. This may lead to significant improvements in the design of individual therapies and therefore to improvements in breast cancer-specific survival [5,6].

Based on the landmark work by Sorlie and colleagues who conducted comprehensive gene expression profiling [3], breast cancer was demonstrated to be multiple biologically distinctive diseases each with its own unique molecular expression pattern [7-9]. In addition, cluster analysis revealed that breast cancer has at least four characteristic molecular subtypes, including ER-positive (luminal), human epidermal growth factor receptor 2 (HER2)-enriched, basal-like, and normal–like [10]. The luminal subtypes can be further divided into luminal A and luminal B, the latter with a higher proliferation rate. The subtypes have specific differences in terms of progression, response to treatment and preferential organs of distant metastasis. Approximately 40% of all breast cancers are luminal A and 20% are luminal B [11,12]. The subtypes can also be roughly separated based on immunohistochemistry [13]. However, based on gene expression, the important molecular distinction between the luminal A and B subtypes is higher expression of ER-related genes and lower expression of HER2 and proliferation-related genes in luminal A[11,12]. In comparison, the luminal B subtype has lower expression of ER-related genes, variable expression of HER2 genes and higher expression of the proliferation-related genes [14].

The HER2-enriched subtype only contributes to 10%–15% of all breast cancers. It is characterized by its low expression of the ER-related genes but a high expression level of the HER2 and proliferation-related genes [15]. Of note, it is not true that all of the molecular HER2-enriched subtypes are the same as the histopathologic-defined HER2 positive breast cancers, and vice versa. That is because not all HER2 amplifications and overexpression are the result of HER2 mutations. Consequently, around 50% of clinically defined HER2-positive tumors are not the HER2-enriched molecular subtype, and are instead characterized as luminal subtypes with HER2-positivity [16].

Around 15%–20% of all breast cancers are categorized as the basal-like subtype, which has low expression of the HER2 and luminal-related genes and high expression of the proliferation-related cluster of genes [17]. It is usually not easy to distinguish between the basal-like subtype and the triple-negative subtype (e.g., ER-negative, PR-negative, HER2-negative), although most basal-like breast cancers are triple-negative, but not all the basal-like cancers are triple-negative. Finally, the normal-like subtype is characterized by a gene expression pattern that is similar to that of normal breast tissue. It remains unclear whether this subtype represents a separate category with clinical significance or a technical artifact of the molecular analysis [16].

Hypotheses have been suggested to explain the intratumoral heterogeneity of breast cancer [18]. According to these theories, luminal lineage-committed progenitors may contribute to form luminal and HER2-enriched cancers, while basal-like cancers may arise from stem-cell-like populations. However, data from gene expression pattern analysis followed by experimental explorations indicate that luminal progenitors also act as precursors to the basal-like subtype. During the tumor forming process, genetic changes or epigenetic events may sequentially switch cellular phenotypes [19-21]. Liu et al. reported that in luminal epithelial cells, loss of *BRCA1* or *PTEN* leads to loss of the luminal cell phenotype, and basal-like cancers form as a result of oncogenetic transformation of these cells [22]. Since not all ER-positive tumors and HER2-enriched or basal-like tumors are the same, we should bear in mind that there might be multiple ways to develop each of tumor types.

Defining the breast cancer evolutionary pathways in humans is challenging, yet meaningful. The characterization would not only provide relevant information for breast cancer risk prediction and chemoprevention studies, but also be important for enriched knowledge of the breast cancer subtypes. Currently, only a few approaches have been implemented in tracing the evolutionary path of human cancer. One method is to collect a large amount of tumors at different stages of progression and analyze the molecular changes. The order of changes can be assembled based on the frequencies at which they are detected at specific progression steps [24,25]. Another approach is to analyze tumors by using next-generation sequencing to detect somatic mutations or alterations at the genetic level. It is based on the assumption that some cells from the early stage of tumor will survive, and their existence and frequency within a tumor may uncover the steps of the tumor's progression [23]. So far, knowledge about the breast cancer cell-of-origin is still limited, and more studies using current sequencing techniques are encouraged in this area.

1.1.2 Intra-tumor heterogeneity

Besides the numerous differences among tumor subtypes, breast cancer cells also display intratumoral heterogeneity. This heterogeneity includes various properties involved in tumorigenesis, for example, tumor invasion, angiogenesis and metastatic potential [26]. Intratumoral heterogeneity may partially reflect non-

genetic mechanisms such as the activity of signaling pathways and the fluctuation of protein expression in response, or adaptive responses that occur during tumor progression. It is believed that intra-tumor heterogeneity can also help to partially explain inter-tumor heterogeneity in breast cancer, since tumors are composed of mixtures of different cancer cells [27].

Cellular differentiation is achieved by epigenetic modifications such as chromatin modification changes and DNA methylation in specific cell types. For certain types of cancers, tumors arise from a group of stem-cell-like or progenitor cells with a preserved capacity to give rise to a more differentiated bulk tumor [28,29]. This model, the so-called cancer stem cell theory, has also been applied to breast cancer, although the validity and interpretation of this model is still not clear. For example, a "gold standard" test currently in use to evaluate cancer stem cells is to perform an orthotropic transplantation assay. Tumor cell clones are transplanted into the mammary fat pad of immunodeficient mice to study their tumor-initiating capacity. Use of this assay as a standard should be limited, because it is very much dependent on the assay model [30,31] and it fails to accurately reflect the behavior of cells in their true physiological environment.

Intra-tumor heterogeneity also poses a major challenge for effective treatment that can eradicate tumor cells thoroughly, because the cellular phenotype of the cell mixture changes constantly during tumor development[32]. Better understating of the tumor heterogeneity maintenance mechanism will be highly useful for improving therapies. Recent studies have also discovered cytokine signaling crosstalk between the tumor microenvironment and the cancer cells. For example, the IL-6/JAK/STAT3 pathway mediates tumor cell growth and survival through paracrine coordination. This knowledge provides the option of targeting this pathway as a new therapeutic strategy for a subset of breast cancers [33,34].

1.2 ESTROGEN RECEPTOR ALFA AND ESTROGEN RECEPTOR BETA IN BREAST CANCER

Estrogens exert their function in the normal breast and in breast cancer through binding to two major estrogen receptor (ER) subtypes, ERα and ERβ [35]. They belong to the nuclear receptor (NR) superfamily composed of ligand-regulated transcription factors [36]. ERα and ERβ are encoded by different genes and exhibit tissue- and cell-specific expression patterns [37,38]. They also display different transcriptional activity after ligand stimulation [37,38]. However, both receptors are co-expressed in a number of tissues and can form either homo- or hetero-dimers to pose their functions[39]. Recent data also indicate that ERs form a complex with the progesterone receptor (PR) to mediate estrogen signaling [40]. In breast cancer, ER subtype-specific expression together with other markers have been evaluated to define different subtypes of breast cancer. Variations in the expression of ERs are associated with different clinical outcomes [41]. Therefore, therapies provided to patients for hormone-dependent cancers based on ER expression are central in improving outcomes.

1.2.1 ER structures and their regulating mechanisms

ERα and ERβ are encoded by two individual genes, ESR1 and ESR2, respectively, which are located on different chromosomes [36,37]. Because they belong to the nuclear receptor superfamily, both receptors contain the evolutionarily-conserved functional and structural domains that are typically observed in NR family members (Figure 1). For example, they all have a six-region structure including domains involved in DNA binding, ligand binding, dimerization and transcriptional activation. The ERs themselves share a high degree of similarity of the sequence in the DNA-binding domains. A motif called a P-box is identical between the two receptors. It is a critical region within the DNA binding domain (DBD) for receptor-DNA recognition and specificity [42,43]. Thus, both receptors have similar specificity and affinity when binding to the estrogen responsive elements (EREs). The ligand binding domains (LBDs) are also conserved in both receptors, and they exhibit similar affinities for the natural endogenous estrogen 17 beta-estradiol (E_2). Nevertheless, ERα and ERβ display distinct affinities for some natural compounds and novel synthetic subtype-selective ligands, and hence there is the possibility for them to be targeted by their selective agonists or antagonists [42-44].

The trans-activating functions of ERs are regulated by two structurally separated but mutually interacting transcription-activation-functions (AFs). The AFs facilitate the receptors to stimulate the transcription of estrogen-related genes and mediate cell and promoter specificity [36,37]. The N-terminal section contains the ligand-independent activation function AF-1, whereas the ligand-dependent activation function AF-2 is located within the LBD at the C-terminus. The AF-1 domains are very active in ERα on estrogen responsive promoters, whereas the activity of AF-1 is minimal in ERβ under the same conditions [38,43].

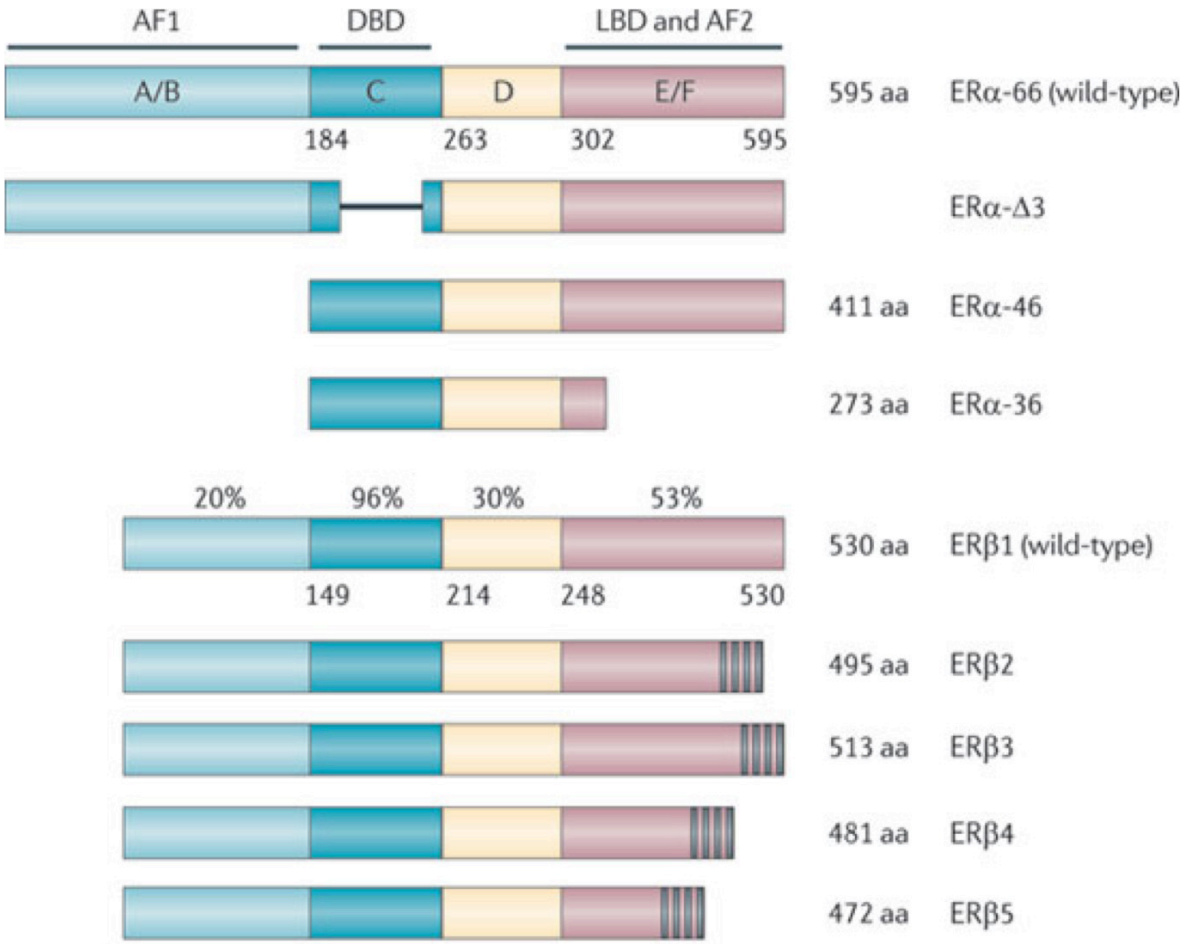

Figure 1. Schematic representation of ERα, ERβ and their splice variants structure.
The structural domains are color labeled and numbers of amino acids are indicated. The percentage of homologies between ERα and ERβ are presented. Functional domains are listed as: activation function 1(AF1), activation function 2(AF2), DNA binding domain (DBD) and ligand binding domain (LDB). From *Thomas, C et al. Nat Rev Cancer 2011.* Reprinted with permission from Nature Publishing Group.

Both ERα and ERβ have several isoforms that are derived from alternative splicing [37,45]. The splice variants have been compared to the wild-type receptors for structural and functional differences in both normal and cancerous tissues. A number of ERα mRNA splice variants have been detected from numerous breast cancer cell lines and patient samples, although wild type ERα is always predominant [45-47]. Some of the ERα variant isoforms have been shown to alter the clinical outcome of patients. For instance, the truncated isoform of ERα has been reported to confer endocrine resistance in breast cancer when co-expressed with wild type [48,49].

ERβ, on the other hand, has far fewer identified isoforms. Only five splice variants have been reported, all as a result of alternative splicing of the last exon of wild type ERβ [50,51]. Most of these variants have been studied in cancers. For example, ERβcx (ERβ2) has been shown to modulate wild type ERβ (ERβ1) and antagonize wild-type ERα transcriptional activity through hetero-dimerization, which is

highly associated with clinical outcomes in breast cancer [51-54]. According to *in vitro* studies, wild-type ERβ and its variant isoform ERβ2 have been characterized as anti-proliferative in ERα-positive cell lines. Once overexpressed, they affect ERα transcriptional complexes, preventing or inhibiting ERα transcriptional activity and modulating the expression of ERα-regulated genes [51,55].

The classical mechanism ERs use to implement their action involves a binding behavior, which means that when ligand binds to the LBD of the receptor, it induces ligand-specific conformational changes of the receptor protein. It will then form either a heterodimer or a homodimer and binds to DNA through the sequence-specific response elements (EREs), which consist of zinc fingers at the DBDs [36,39]. Once bound to EREs, the receptor dimers will recruit co-activators or co-repressors of different transcriptional factors depending on the shape of the ligand-receptor complex. The receptor activity is influenced by these complexes, which either activate or repress downstream gene transcription. Co-regulatory proteins are recruited in the process of complex formation, such as histone acetyltransferases that mainly work by modification of chromatin structure, and ubiquitin ligases that are recruited for the enhancement of transcription [56,57]. After the initiation step, post-translational modifications will occur, among which, methylation or acetylation leads to further activation of the receptors and promotes the dissociation of the complex or the simultaneous ubiquitylation followed by degradation of the ERs [39,42,43].

Notably, in the absence of ligands, ERs can sometimes still elicit their transcriptional responses. For example, insulin- like growth factor 1 receptor (IGF1R) or epidermal growth factor receptor (EGFR), which are the members of the hyperactive growth factor receptor family, can activate ERs by stimulating protein kinase cascades in the absence of ligands [58]. New evidence indicates that populations of ER subtypes located on the membrane or in the cytoplasm can mediate the rapid responses of estrogens [59]. Together with the involvement of other signaling pathways such as MAPK, PI3K, ERBB2, EGFR, SRC, IGFIR and G proteins, the activation of key components by ERs can be observed within 3-15 minutes of estrogen treatment, and these further trigger other cascades to regulate gene expression by activating other transcriptional factors [60-62]. Methylation of ERα by estrogen is also frequently observed in breast cancer. This results in the formation of complexes that are mainly composed of ERα, PI3K and SRC. Additionally, it also influences gene transcriptional events by activating AKT [44].

1.2.2 Different transcriptional responses

Although structurally homogenous, indicating that ERα and ERβ will share similarities in mechanical actions, it is not surprising to find functional differences in transcription responses elicited by the two different ERs. This is validated by microarray analysis of endogenous ER genes in breast cancer cells, where expression patterns reveal that the two ERs regulate some genes in common, regulate different genes and regulate some genes at different expression levels [63].

Moreover, *in vitro* experiments suggest that ERα and ERβ display divergent affinities and transcriptional responses to their specific selective ligands [37]. Even when binding to the same ligand, they have different response effects or signaling cascades that activate receptors at separate locations within the cells. 17β-estradiol serves as a classical agonist for both ERs, and can induce conformational changes in the receptors that result in different binding selectivity. In ERα, it can provoke the binding of only co-activators compared to ERβ, which recruits both co-activators and co-repressors [65]. In contrast with 17β-estradiol, tamoxifen acts as a classical antagonist to ERα by recruiting co-repressors after binding to the receptor, but it does not have the same effect when binding to ERβ [64,65]. This implies a relative balance between co-activator and co-repressor expression that can affect the activity of agonist and antagonist in the cells. Additionally, other studies reported that ERα and ERβ differentially regulate MYC, a nuclear transcription factor with mitogenic effect that can induce cell cycle progression [66,67].

1.2.3 Estrogen and ERs in the normal mammary gland and in breast cancer progression

To date, our knowledge of the role of ERs in human mammary gland development is still lacking in fundamental data. Only pieces of key information are available to fill in the gaps in our knowledge of estrogen signaling. Based on previous observations, ERα is mostly located in the inner layer of epithelial cells or the external layer of myoepithelial cells lining the intralobular ducts in premenopausal women. In comparison, the distribution of ERβ is more widespread, in both epithelial and stromal cells [68]. In postmenopausal women, ERα expression is only found in less than 10% of mammary epithelial cells whereas ERβ is expressed in more than 50% of epithelial cells. The stromal component in postmenopausal women also expresses higher levels of nuclear ERβ but not ERα [69]. Based on *in vivo* studies, ERα has been proven to be responsible for cellular proliferation as an indirect effect after estrogen stimuli [70]. ERβ represses proliferation and is pro-apoptotic when it is overexpressed *in vitro* [37,71]. This indirect effect of ERα on promoting proliferation has been reported to occur through a paracrine mechanism involving ERα-positive cells[70], although this has only been demonstrated in mouse models. A number of paracrine mediators that might account for the effects of estrogens have been identified from other studies. Among these, the receptor activator of amphiregulin is suggested to be a critical paracrine mediator for ERα[72].

In breast cancer, a few reports have concluded that ERα might regulate cancer cell migration and invasion. ERα represses nuclear factor (NF)-κB and activator protein 1 (AP-1), but activates and increases E-cadherin expression by down-regulating its repressors to further control the epithelial-mesenchymal transformation (EMT)[73,74]. Similar to ERα, ERβ also mediates cell migration. In prostate cancer, down-regulation of ERβ is found to induce EMT, which is consistent with the fact that many prostate cancers experience a loss of ERβ. When ERβ is activated by its specific ligand 3β-adiol, a metabolite of

dihydrotestosterone, the expression of E-cadherin will enable the prostate cancer cells to sustain an epithelial phenotype and repress invasion[75]. In addition, knock-out of ERβ can decrease E-cadherin expression in mouse mammary epithelial cells [76]. The same observations may apply to the increased expression of integrin α1 and β1 due to ERβ, which enhances breast cancer cell adhesion in culture [77]. These facts all support the idea that ERβ maintains a differentiated epithelial phenotype through the inhibition of EMT.

1.3 ENDOCRINE THERAPY FOR BREAST CANCER AND POSSIBLE MECHANISMS OF RESISTANCE

In the previous section, we have discussed breast cancer as a heterogeneous disease composed of multiple subgroups with their distinctive molecular backgrounds, different risk factors for incidence and disease progression and preferred organ sites for metastases. Across all of the breast cancer types, at least 70% of tumors are classified as ERα-positive [78]. This phenotype has been explained by the sustained exposure to endo- or exogenous estrogen through estrogen receptors [79]. Thus, interfering with estrogen and receptors has been a cornerstone of treating breast cancer for over a century. Synthesis of estrogen-ER binding site competitive inhibitors first occurred in the 1970s, including the selective estrogen receptor modulator (SERM) tamoxifen, which successfully increased the survival rate of breast cancer patients. Today, these agents are still used as primary therapy for breast cancer [80]. Tamoxifen used as adjuvant therapy can reduce by one-third the number of annual breast cancer deaths and it halves the recurrence rate. It contributes to a significant decrease (around 30%) in breast cancer mortality [81]. Other new, effective endocrine synthetics have been developed in recent years to target estrogen actions, such as the aromatase inhibitors and ER signaling antagonists [82,83]. The aromatase inhibitors act by blocking the enzyme aromatase, which catalyze the hormone androgen into estrogen [82,84]. Fulvestrant, a complete ER antagonist, can accelerate degradation of the ER and serve as second-line therapy for patients who failed in the previous endocrine therapy [85,86].

However, a crucial issue about tamoxifen administration is that 33% of women treated with tamoxifen for 5 years will develop a recurrence within 15 years [81], which means that around 25% of all breast cancers become endocrine-resistant. This observation raises two major challenges for improvement of breast cancer treatment: 1. Identifying new therapeutic targets against endocrine-resistant cancer, and 2. Developing more specific biomarkers for predicting therapeutic response to endocrine therapy.

1.3.1 Molecular mechanisms of resistance

The primary mechanism of intrinsic resistance to tamoxifen is loss of ERα expression. Other mechanisms are supposed to account for acquired resistance to tamoxifen due to the long-term exposure [87]. Many studies reporting these potential molecular mechanisms were based on observations derived from ERα-

positive breast cancer cell lines or variants of the cell lines selected for adaptation to sustained exposure to estrogen or absence of estrogen. Therefore, conclusions drawn from such models have several limitations since cell lines are induced to be tamoxifen resistant *in vitro* rather than exhibiting the actual resistance developed in breast cancer patients. The accuracy and validity of the proposed mechanisms should also be questioned since the *in vivo* condition with an epithelial–stromal tumor microenvironment probably modulates tamoxifen sensitivity [88,89], and it is unlikely that ERα positive breast cancer cell lines can truly reflect the range of ER-positive phenotypes that can develop *in vivo*. In spite of the potential limitations, cell line models are still widely used to study the candidate genes involving in estrogen signaling or regulation of cell proliferation. The concepts and hypotheses developed are still important in providing indications for tamoxifen resistance in patients and provide a basis for modeling new therapeutic approaches. Deregulation of estrogen signaling is a major mechanism for tamoxifen resistance, although other mechanisms contributing to the resistance should also be investigated, such as, cancer cells that have developed alternative proliferation and survival mechanisms [87,90].

1.3.2 Targeting ER signaling and co-regulators

ERα expression is currently the principal biomarker and indicator of response to endocrine therapies like tamoxifen, since ER-negative breast cancers rarely respond to them. Earlier publications tried to state that the potential mechanisms of acquired resistance were due to ERα mutations and loss of ERα expression. However, later validation studies found that ERα mutations only appeared in fewer than 1% of ER positive tumors [38,91,92] while only 15%-20% of tamoxifen-resistant tumors were lacking ERα expression [93]. Recently, Shi et al. reported that in the presence of ERα, its truncated variant ERα36 was associated with a reduced response to tamoxifen [49]. ERβ and other variants also play potential roles in tamoxifen responsiveness, especially when ERα is absent [94]. In addition, estrogen-related receptor (ERRγ) was found to be overexpressed in lobular invasive breast cancer and can induce tamoxifen resistance [95].

In the previous section, we have described the mechanisms whereby ERα regulates gene expression. This occurs mainly through protein-protein interactions with other transcriptional factors such as NF-κB, Ap1 and specificity protein 1 (Sp1). Of these, NF-κB and Ap1 transcriptional activity are reported to be correlated with endocrine resistance [96-98]. Post-translational modifications including sumoylation, phosphorylation and methylation also affect ERα function[99] by changing its interactions with other transcriptional proteins and cytoplasmic signaling components [100,101].

1.3.3 Receptor tyrosine kinase signaling

Some early observations identified the crosstalk between ER signaling and receptor tyrosine kinase signaling by showing that the expression of ER correlates with the epidermal growth factor receptor (EGFR) family members including

EGFR and ERBB2 [102]. *In vitro* studies showed that growth factors and insulin like growth factors can modulate tamoxifen susceptibility in breast cancer cells [103]. Increased expression of those receptors could drive tamoxifen resistance [104-106] by activating their downstream signaling pathways, particularly the effectors in the PI3K pathways [107-109].

The deregulation of these signaling pathways can be classified into two groups. First, genetic or epigenetic modifications might take place. Methylation or loss of PTEN (a tumor suppressor inhibiting the PI3K pathway), amplification of ERBB2 and mutations of PIK3CA (it encodes a catalytic subunit of PI3Ks) can all contribute to the deregulation [92,110]. Second, abnormality of upstream regulators of these pathways might occur. For instance, loss of PTEN expression and ERBB2 overexpression are associated with AKT activation. IGFR1 and ERBB3 are also found to be activated as a consequence of the absence of PTEN [92,110]. Although the mechanism behind tamoxifen resistance mediated by these factors has not yet been fully elucidated, there are some clues as to the cause of the resistance. For example, ERK activation decreases ERα expression, ER-mediated repression of EGFR subsequently activates mitogenic signaling cascades, and ligand-independent activation of ER and its co-activators through phosphorylation and constitutive activation of survival signaling further inhibit apoptosis [44]. ERBB2 overexpression is one of the well-established mechanisms of endocrine resistance. It is known that amplification of *ERBB2* and loss of transcriptional repressors are mainly responsible for increased expression of this receptor[111-113]. On the contrary, ERBB2 repression is correlated with increased survival after tamoxifen treatment [112]. In addition, the members of tyrosine kinases in SRC family, particularly downstream targets of SRC are implicated in tamoxifen resistance in breast cancer [114-116].

1.3.4 Cell cycle regulators

Neo-adjuvant and adjuvant endocrine therapies have been supported by experimental model systems with clinical correlations as being both cytostatic and cytotoxic [117]. These therapies can lead to decreased proliferation and increased cell apoptosis. In cell culture, estrogen antagonist treatment with agents such as tamoxifen or aromatase inhibitors can result in a G1 phase-specific cell cycle arrest, which leads to the activation of the cellular stress response and apoptosis in breast cancer cells, and consequently a reduced cell growth rate [118]. Not surprisingly, *in vitro* data has shown that cMYC, cyclin E and cyclin D are crucial for the anti-estrogen effects on cell cycle progression, especially in controlling the G1 phase arrest and apoptotic initiation. Once their expression levels become aberrant, endocrine treatment resistance might occur that further contributes to reduced tamoxifen responsiveness in breast cancer patients [119-124].

1.4 BREAST CANCER STEM CELLS AND THEIR IMPACT ON BREAST CANCER PROGNOSIS

1.4.1 The cancer stem cell hypothesis

Even though it was developed only recently, the hypothesis of cancer stem cells (CSCs) has had considerable impact on cancer research (Figure 2). With the current advanced new techniques and animal models, direct tests and validated results have been quickly produced from cancer stem cell research. The major idea of this hypothesis is that solid tumors contain a unique subset of cellular components displaying normal stem cell properties. These include: the capacity to self-renew, the ability to differentiate under certain stimuli, active telomerase expression, active anti-apoptotic pathways and the potential to migrate and metastasize [125]. CSCs were first discovered by Dick and colleagues in human acute myeloid leukemia [29,126]. Gradually, CSCs existence was identified from other types of cancer. Although they only make up a small proportion of the tumor mass, CSCs are highly potent in their ability to give rise to other types of tumor cells and create hierarchically integrated tumors [28,29,126-128]. Accumulating data suggests that CSCs are resistant to conventional therapies and play an important role in mediating tumor metastasis and relapse [129-132]. Hence, the CSCs hypothesis provides a plausible explanation for treatment failure and attracts increasing attention in current research. The complexity and heterogeneity of cancer indicates that the mechanisms for resistance and recurrence caused by CSCs vary depending on the type of cancer. Therefore, specific target therapies against different types of tumors may be required.

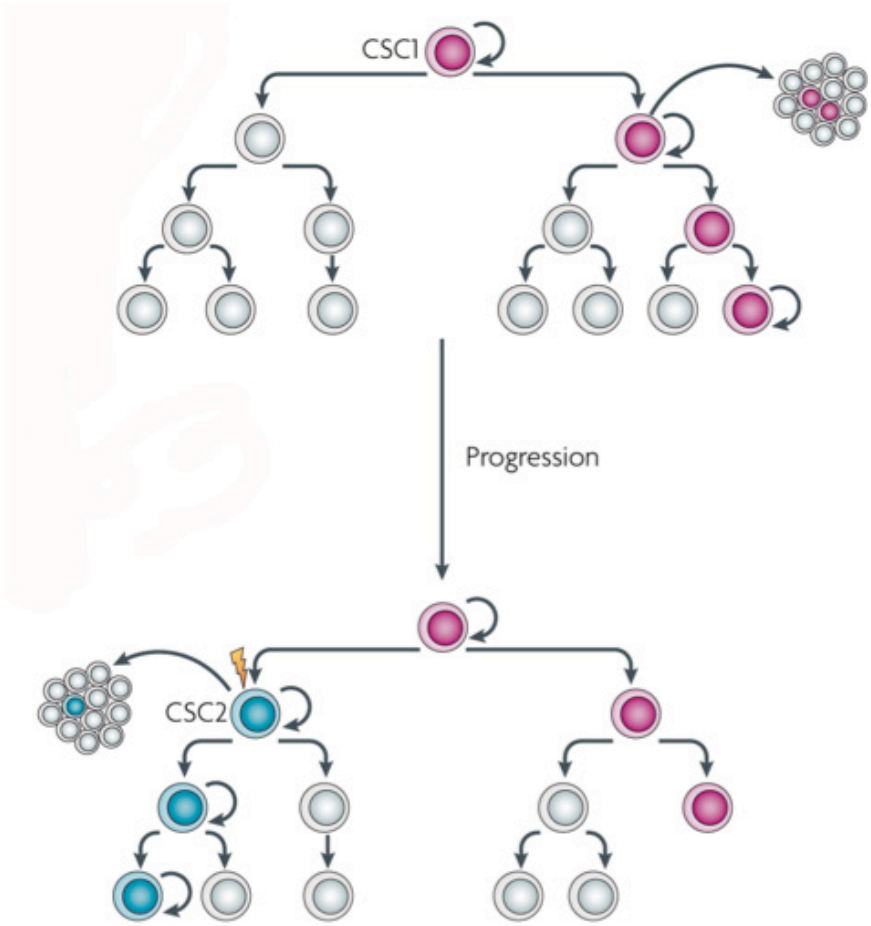

Figure 2. The hypothesis of tumorigenesis governed by CSC. CSC generates a bulk tumor based on its self-renewal properties and proliferative potential. Initially, tumor growth might be driven by a certain CSC1, along with the tumor progression, another distinct CSC2 may evolve from CSC1 and take over the capacity to form tumor in the later stage. From *Jane E, V. et al. Nat Rev Cancer 2008.* Reprinted with permission from Nature Publishing Group.

1.4.2 Identify BCSCs and their roles in breast cancer biology

The applicability of the CSCs hypothesis in solid tumors remains in heated debate. In 2003, CSCs from human breast cancers were first identified by Al-Hajj and collaborators [28]. Thereafter, CSCs have been identified in most of the solid cancers, including brain, colon, pancreatic, head and neck, and others [127,128,133]. Following the work of Al-Hajj et al., researchers isolated subpopulations of cells from primary tumors and pleural effusions from breast cancer patients. Only the cells carrying the phenotype of $ESA^{+}CD44^{+}CD24^{-/low}$ were able to reform tumors when transplanted in as few as 100 cells into the mammary fat pad of NOD/SCID mice, while larger populations of other types of cells retained their non-tumorigenic properties [28]. Therefore, the cell surface markers $CD44^{+}/CD24^{-}$ were suggested as a combination that can be used to identify breast cancer CSCs (BCSCs). Later on,

Ginestier et al. described another small subset of $CD44^+/CD24^-$ cells with an additional ALDH1-high phenotype that were highly tumorigenic, proven by showing that injection of only 20 cells into NOD/SCID mice was sufficient to initiate tumor formation[134]. The ALDH1-positive cells not only recapitulated the heterogeneity of the parental tumor, displaying non-tumorigenic components, but also repeated this process upon serial passaging in the mouse model. This recreation of the original tumor demonstrated both their differentiation and self-renewal potential as stem cells. Further research has focused on the clinical implications of ALDH1 expression in breast cancer, indicating that its presence correlates with poor prognosis in breast cancer patients [134,135]. Recent publications also added additional information about the $CD44^+/CD24^-$ population *in vivo*, showing that they are enriched in basal-like tumors as well as in BRCA1 tumors [136], and that they indicate a poor prognosis[137,138].

Notably, several articles have mentioned successful generation of BCSCs from either breast cancer patient-derived material or from breast cancer cell lines by using verified selectable markers [139,140]. In the other hand, the estimation of BCSCs frequency varies extensively in different cell lines. For example, the breast cancer cell line MDA-MB-231 has been reported to contain 85% $CD44^+/CD24^-$ cells, but only 0.88% are ALDH1-positive, and the MCF7 cell line has no $CD44^+/CD24^-$ cell populations but 0.2% of the cells are ALDH1-positive[139,141]. This huge difference signifies that none of these identified BCSCs markers are universal to breast cancer. Thus, continuing the search for novel markers or combining new markers with the existing markers will presumably improve the isolation of BCSCs and lead to understanding of their implications in breast cancer progression, relapse and resistance.

1.4.3 The role of BCSCs in metastasis

Breast cancer is highly treatable with a very promising 5-year survival rate, if it can be detected in the early stages. However, good outcomes become rare once the disease metastasizes [142,143]. The process of metastasis has been well established as a stepwise procedure. In general, the cancer cells will first detach from the primary tumor and enter the bloodstream or the lymphatic circulation via intravasation. Carried by the circulatory system, surviving cells will reach a secondary site, and then extravasate into the tissue. These cancer cells are highly potent in initiating colony growth, which forms a micro-metastasis followed by subsequent angiogenesis to promote macro-metastasis progression[142,144,145].

Interestingly, metastatic lesions are lethal, yet it has been proven to be a highly inefficient process. Luzzi and colleagues used an *in vivo* video microscopy to observe the process. Although exemplified by melanoma, a highly metastatic tumor, only 0.02% of the injected cells could successfully induce a metastatic cascade in the targeted organ [146]. The incidence of cells able to form metastases as reported previously corresponds to the proportion of CSCs in cancers. In fact, in an eloquent review by Croker and Allan, they presumed that in breast cancer the inefficiency is on account of the absence of a favorable microenvironment for a secondary growth in distant tissue, hence preventing the majority of circulating

cancer cells from anchoring [147]. However, the potency of CSCs makes them ideal metastasis-initiating cells, as they exhibit properties like self-renewal, capacity to inhabit many different microenvironments and inherent resistance to apoptosis and conventional treatments.

Meanwhile, proof that BCSCs are responsible for breast cancer metastasis has been collected from two perspectives. First, it has been shown that the EMT is coupled with the formation of BCSCs [148,149]. An EMT is an initial step in the metastatic progress. Chemotherapy induces residual breast cancer cells to adopt both a BCSC phenotype and EMT features. Normal mammary epithelial cells also display enhanced BCSCs properties when undergoing an EMT [149]. Regulators of EMT expression also drive non-tumorigenic cancer cells towards a tumorigenic status[129,150,151]. Moreover, signaling pathways involved in MET, the reverse process of EMT, which is an essential step in the last stage of metastasis, also induce stem-cell-like characteristics in breast cancer cells [152]. Additionally, $CD44^+/CD24^-$ defined BCSCs subpopulations not only boost the incidence of metastasis when administrated through mice tail veins [153], but also co-express EMT markers and furthermore reduce the burden for metastatic events [149].

Breast cancer metastases that develop at different sites seem to always carry the BCSCs $CD44^+$ profile in mouse xenograft studies [154-156]. The metastatic lesions at orthotopic sites have been confirmed to express high levels of CD44, which was experimentally demonstrated to play a central role in the attachment of metastatic breast cancer cells to the bone marrow endothelial cells when interacting with osteopontin [156]. A study on a spontaneous lung metastasis also found the generated tumor had a similar rate of CD44 positivity as compared to the primary tumor [157]. In addition, we and other research groups have described BCSCs that are ER- independent of parental tumor ER status. It is not rare that ER- metastatic cancers are found in ER+ patients [158,159], which demonstrates the metastatic potential of ER- BCSCs or alternatively provides a possible explanation of subtype shifting. Expression of the ALDH1 isoform ALDH1A3 is also associated with metastasis [160,161].

1.4.4 The role of BCSCs in conventional therapy resistance

Studies in recent years have shown that CSCs are closely related to poor clinical outcomes in different cancers [162-164]. In breast cancer, the frequency of BCSCs has been demonstrated to be associated with the aggressiveness of primary breast cancer [165]. In experiments involving orthotopic transplantation of different types of breast cancer cells, poorly differentiated tumors containing higher BCSCs content [165,166]. Another batch of studies showed that triple-negative breast cancers that can also be classified as claudin-low subtypes have a much worse prognosis due to a clear enrichment of the BCSCs signature compared to the luminal types of cancer [167,168]. Moreover, accumulating evidence supports the existence of BCSCs leading to failure of conventional systemic therapies [169-171].

1.4.5 Major pathways utilized by BCSCs to mediate treatment resistance

In order to better recognize the nature of BCSCs and effectively target this unique cell type in future therapies, it is important to first understand the details about the different pathways BCSCs utilize to maintain their self-renewal, survival and slow proliferating features.

1.4.5.1 Hedgehog signaling

Hedgehog (Hh) was first identified during Drosophila embryonic genetic pattern screening [172]. It is critical in developing embryos to direct organogenesis, regulating cell proliferation and differentiation. Although its significant function in postnatal mammary gland development is under debate, Hh signaling has been shown to play an important role in normal breast stem cells. Paracrine Hh signaling can expand and stimulate progenitor population proliferation in the mouse mammary gland [173,174]. Liu et al. and Moraes et al. reported that Hh signaling activation could promote mammosphere formation in normal mammary stem cells, whereas treatment with its inhibitor cyclopamine can reverse this effect [175,176].

Giving the evidence from above, it is not surprising to find Hh also involved in malignant BCSCs in the mammary system. Hh signaling was found to be highly activated within the subpopulation of $CD44^{+}/CD24^{-}$ cells from patient-derived BCSCs. In a mouse model, activation of Hh induced mammosphere formation in p53-null mouse mammary tumor through the polycomb protein BMI1, which is usually overexpressed in the $CD29^{H}/CD24^{H}$ subset of cells, and a reduction of BMI1 expression led to decreased mammosphere formation[175,177]. Moreover, regulators in the Hh signaling pathway, for example GLI1, GLI2 (GLI transcription family members), and BMI1 are expressed at higher levels in $CD44^{+}/CD24^{-}$ BCSCs compared to bulk tumor cells. Recently, GLI1 as a downstream mediator of Hh signaling has been shown to stimulate triple-negative breast cancer initiation [178] and it is active in luminal breast cancers, suggesting it is required for BCSCs to self-renew [179]. These results indicate that targeting Hh signaling components is one approach to disable BCSCs.

1.4.5.2 Notch signaling

In mammals, there are four transmembrane Notch receptor proteins (Notch 1-4). Activation of the Notch signaling pathway occurs when the Notch receptors 1-4 bind with their ligands in the cells. Binding leads to proteolytic cleavage cascades, caused by internal cleavage by gamma-secretase as well as by ADAM metalloprotease family members [180]. Cleavage results in the translocation of the NICD (Notch intracellular domain) into the nucleus where it forms a trimeric complex with the DNA-binding protein [181]. This further induces the expression of Notch downstream target genes including Hey, cyclin D1, c-Myc and Hes [182].

Notch signaling in breast cancer was demonstrated by the identification of a proto-oncogene that was being abnormally expressed in response to Notch-4 by

retroviral insertions in mouse mammary cancers [183]. An activated Notch pathway will lead to the formation of mammospheres in sequential passages from normal mammary stem cells while specific inhibitors can suppress the process [184]. In addition, both Notch1 and Notch4 antibodies can decrease the mammosphere formation efficiency of breast cancer cells from patient materials or patient-derived xenografts (PDX) in mouse models[185,186], suggesting the importance of the function of Notch in both normal stem cells and cancer stem cells in the breast. Importantly, one study showed Notch4 activity increased 8-fold in the $CD44^{+}/CD24^{-}$ BCSCs subpopulation, and inhibition of Notch4 reduced the BCSCs frequency in breast cancer cell lines along with complete inhibition of tumor growth[187].

Another study observed activated Notch signaling in ER- cells, although it is inactivated in ER+ cells even when estradiol is present[188], providing a potential target for hindering Notch signaling to attenuate endocrine therapy resistance in the putative BCSCs, which are defined as having an ER- status. From a more recent study, in basal like breast cancer, where the expression of Notch ligand JAG1 is induced by NF-κB, Notch signaling in non-tumorigenic cancer cells could also be triggered, leading to the expansion of the BCSCs population[189]. Notably, Notch1 activity was shown to be lower in BCSCs compared to more differentiated cancer cells. This might imply that different Notch receptors play a role in the regulation of CSCs and other cancer cells in the breast [132].

1.4.5.3 Cytokine and IL-8/CXCR1/2 signaling

Recognizing the cytokine signaling network is essential to understanding tumor biology, especially in cell integrations within a tumor microenvironment. Rather than simplistically viewing the solid breast tumor as a sheet of homogenous epithelial cells cultured *in vitro*, tumors in physiological conditions consist more of a hierarchical complex composed not only of epithelial cells, but also endothelial cells, fibroblasts and other cell types that communicate with each other through growth factors and cytokines. Interfering with this network is a growing interest for drug discovery, because disrupting this network seems to be highly effective at completely eradicating tumors in acute myeloid leukemia (AML) in mouse models [190-192].

Several cytokines including IL-6 [193], IL-8 and the CXCR1 receptor were shown to be involved in regulating BCSCs. CXCR1 belongs to the G-protein-coupled receptor family. Its expression is higher in ALDH1-positive cells from several breast cancer cell lines [139]. IL-8 treatment can directly stimulate an increase in the ALDH1-positive fraction and mammosphere formation in a panel of breast cancer cell lines [139,194]. Ginestier and colleagues demonstrated that in breast cancer cells, the proportion of cells that are CXCR1-positive overlaps with the ALDH1-positive subpopulation. It is possible that BCSCs could be depleted when subjecting to treatment with CXCR1 inhibitors [195].

As the most-studied CXCR1/2 ligand, IL-8 has been reported to be expressed in response to signals send from BCSCs, and mesenchymal stem cells secret IL-8 to

reinforce the message from BCSCs [33]. Recent publications have also shown that stimulating IL-8/CXCR can induce mammosphere formation, and the process is partially mediated by EGFR/HER2 signaling [194]. Antagonizing IL-8/CXCR seems to be a rational strategy for targeting BCSCs, and clinical trials using antibodies against IL-8 have shown promising results in treating inflammatory diseases [196]. However, the benefits from inhibiting IL-8 should not be exaggerated, since CXCR1/2 activation can be achieved by binding to a broad range of other ligands [197]. Hence, when developing new therapeutic strategies the focus should be on blocking CXCR function for better specificity, or on using combination treatment with current drugs that provide an additive effect.

1.4.5.4 AKT as a central node in BCSCs signaling pathways

AKT is a central mediator in the phosphatidylinositol 3-kinase (PI3K), Wnt and other signaling pathways and it is critical in regulating metabolic homeostasis. The AKT pathway is activated by binding of an extracellular ligand to a receptor tyrosine kinase (RTK) in the plasma membrane, causing phosphorylation of PI3K, which leads to the membrane interaction with phosphoinositides that act as second messengers to recruit and fully activate AKT [198]. Phosphorylation of AKT triggers downstream cascades of different proteins that regulate diverse biological functions, such as cell proliferation, survival, apoptosis and mobility [198]. The components of AKT signaling are widely found to be gain-or-loss of function mutations in human cancers. All of these deregulations lead to neoplastic transformation [199]. PTEN is a lipid phosphatase that is upstream of AKT [200], which functions as a tumor suppressor that opposes PI3K to phosphorylate AKT [201,202]. Loss of PTEN has been observed in many tumors, resulting in the accumulation of PIP3, a product of PI3K, that can be dephosphorylated by PTEN which leads to further activation of a signaling cascade such as AKT, mTOR and phosphatidylinositol dependent kinases [200,201]. Activated AKT promotes cell cycle progression and down-regulates pro-apoptotic factors. In mouse models, it has been shown that the PI3K/AKT pathway can mediate stem cell maintenance and malignant transformation in a variety of tissues [203-206].

In normal mammary gland stem cells and breast cancer, the role of PTEN is documented from different observations. In mammary epithelial cells, PTEN deletion induces precocious development and neoplasia in the mammary gland [207]. In normal breast tissue from humans, knockdown of PTEN increases activation of the Wnt pathway, AKT activity and boosts formation of mammospheres [208]. In different breast cancer cell lines, PTEN knockdown again increases mammosphere formation and drives tumor initiation in NOD/SCID mice. In normal mammary cells, knockdown of PTEN induces disorganized hyperplastic lesion generation in mouse models [208]. Accordingly, the AKT inhibitor results in formation of fewer mammospheres *in vitro* and reduces hyperplastic lesions, tumor growth and formation of secondary tumor dramatically *in vivo.* In consequence, Wnt signaling activation increases mammosphere formation in patient derived material [208-210].

1.4.6 Metabolism signaling pathways

Under normal physiological conditions, glucose is the primary source of energy for cellular respiration. In glucose metabolism, around 70% of ATP is synthesized by oxidative phosphorylation and the remaining 30% is by glycolysis, though the ratio of ATP generated from each process varies in different cell types, under different growth conditions and in different microenvironments [211]. In most solid tumors, an hypoxic core is usually found. Therefore in cancer cells, it is common to observe a metabolic switch towards enhanced glycolysis over respiration in defiance of functional oxidative phosphorylation [212]. Meanwhile, if the oxidative phosphorylation machinery is restricted by suppression of mitochondrial respiration or hypoxia, lactate fermentation is recruited to compensate for the cellular energy generation [213].

Cancer cells require an enormous amount of energy in order to rapidly proliferate. The altered metabolism during tumor progression that cancer cells manifest to meet energy needs is called the Warburg effect [214]. The process involves a metabolic shift from oxidative respiration to glycolysis and lactate fermentation even with the presence of oxygen, and it was originally interpreted as an increased glycolysis to meet energy demand in tumors as a result of mitochondrial defects with disrupted mitochondrial respirations. However, hypoxia can diminish the glycolytic pathway inhibited by oxygen and further enhance anaerobic glycolysis in cancer cells [212].

However, it was later discovered that cancer cells can utilize glycolysis for energy demand even with normally functioning mitochondria. Due to the much lower efficiency of converting glucose to lactate as compared to directly metabolizing glucose through the mitochondrial oxidative phosphorylation, a high rate of glucose uptake is necessary to provide the increased energy consumption to support rapid cell proliferation. ATP generation depending on fermentative metabolism is a long term reprogramming process in cancer cells. In addition, acute repressive signaling cascades suppressing mitochondrial function are also commonly observed [211-213]. Compared to normal cells, this metabolic shift towards glycolysis and suppression of mitochondrial respiratory machinery is irreversible, and alterations of glucose metabolism supplying additional energy to support tumor growth have been reported in many studies. This suggests that inhibition of glycolysis could lead to reduced tumor cell proliferation and prevent tumor progression [215].

In humans, normal embryonic stem cells display a glycolytic metabolism, possibly due to a mitochondria variation. Based on some studies, highly undifferentiated cells such as CSCs are able to shift between glutaminolysis and aerobic glycolysis [216,217]. Feng and colleagues recently showed that enriched populations of BCSCs rely more on glycolysis than do non-tumorigenic cells, based on observations of mitochondrial number differences and repressed expression of the key mitochondrial enzymes [218]. The glycolytic phenotype is distinctive in CSCs with an overexpression of most of the glycolytic enzymes. Lactate dehydrogenase and its isoforms are found to be highly expressed under normal conditions, and are

upregulated under c-MYC activation and hypoxia, which facilitate moving away from oxidative metabolism [219].

However, there are still some cancer cells that rely on oxidative phosphorylation rather than glycolysis. These different metabolic profiles exhibited by CSCs are dependent on their degree of differentiation and tissue of origin [219]. Liver cancer cells, for example, are usually highly undifferentiated and tend to rely more on glycolysis than differentiated tumor cells [220]. Normal glioma stem cells have been shown to consume less glucose and yield less lactate than their related cancerous cells [221]. Zhang et al. showed that non-small cell lung cancer stem cells selectively upregulate glycolytic genes [222]. Despite the intrinsic needs of CSCs, exogenous factors also influence both metabolic processes and cell fate [219].

The concept of niche, or the resident microenvironment, is an essential factor that distinguishes CSCs from normal stem cells. Niche functions as a source of molecules, which either activate or inhibit signaling transactions. In normal tissues, the microenvironment for stem cells is known to retain the balance between self-renewal, proliferation and differentiation [223-225], whereas in CSCs, the required tumor microenvironment is altered towards maintaining the proliferating signals [226,227].

The idea that tumor microenvironment plays a central role in tumor initiation and progression has been widely accepted. Alterations in the microenvironment can be achieved through stromal or immune cells, alternation of extracellular modifications, as well as changes in the oxygen concentration [228]. Typically, the tumor niches are characterized as having low oxygen concentrations and a glycolytic-mediated phenotype, which is partially promoted by the HIF-signaling pathway. During the cancer initiation phase, the niche becomes hypoxic and favors the activation of genes associated with stem-cell-like profiles, such as *Notch*, *Nanog*, *Sox2* or *Oct4*, together with genes associated with the glycolytic switch [229].

In solid tumors, the capacity of CSCs to modulate the tumor microenvironment has led to the proposal that CSCs adaptation to hypoxia may lead to the glycolytic shift and finally induce acidity changes in tumor niches. In brain tumor models, local pH measurements shift from 7.1 to 6.8 in normal tissue versus tumor tissue [230]. The acidification of the microenvironment would in turn modify the acidity of proteases, which modulate extracellular matrix degradation. These changes of acidification promote maintenance of the CSCs phenotype maintenance in bulky tumors. Several studies have also suggested that through activation of enzymes involved in the extracellular matrix [231] and HIF-dependent pathways, hypoxia can promote and facilitate metastasis of cancer [232]. In clinic practice, there is an association between the presence of a hypoxia core in tumors and a poor prognosis for patients [232-234].

1.5 MODELING BREAST CANCER

Breast cancer is not a single disease. Instead, it is a mixture of breast diseases having diverse genetic variations, histopathological profiles and clinical outcomes.

For many decades, our knowledge and understanding of breast cancer biology has been based on experimental model systems. A major question that needs to be considered is whether the experimental models really recapitulate the true forms of breast cancer. Due to its heterogeneity and complexity, no single model would be able to completely represent or mimic all aspects of breast cancer. Thus, when modeling breast cancer to investigate clinical consequences, we should always keep in mind key questions such as how to choose the current existing model and how to improve preclinical models based on the research question. It is necessary to develop new models for treatment evaluation in terms of metastasis and the mechanisms underlying secondary tumor progression, which is the principal cause of mortality of breast cancer.

1.5.1 *In vitro* breast cancer cell line versus primary human breast cancer cells

Recently, several studies have investigated gene genomic alterations and expression profiles by comparing primary breast cancers and various breast cancer cell lines [10,235-237]. Not surprisingly, the findings from these studies indicate that none of these cell lines alone is representative of breast cancer. However, using a panel of cells lines can represent the heterogeneity observed in primary breast cancers. A comprehensive study conducted by Neve et al. reported that many genomic abnormalities are the same between a panel of 51 breast cancer cell lines and primary tumors [237]. Therefore, the establishment of cultured cell lines to model primary breast cancer can be adapted to study the common genomic aberrations.

Nevertheless, the differences between the cell lines compared to the primary tumors are still crucial for potential usage. For instance, not all of the common subtypes of breast carcinoma are represented, including luminal A, luminal B, HER2 enriched, triple negative and basal like subtypes. Additionally, the frequencies of copy number abnormalities differ profoundly between luminal and basal subtypes of primary tumors. Notably, cell lines fail to display the features of the luminal subtypes [238]. Thus, although investigators prefer to classify cell lines into luminal and basal subtypes, this is less-than-accurately representative of breast cancer biology in the clinic. This suggests that caution needs to be used when using the cell line model to study breast cancer.

The reason why a difference exists between cell lines and primary tumors can probably be explained by the methods used to generate cell lines from breast cancer patients. They are usually obtained either from pleural effusions or from advanced stage tumors, therefore the most malignant variants that could be adapted to cell culture represent the cell lines. Notably, gene expression profiles have shown that primary tumors and secondary tumors from metastasis share high similarities [239,240]. This suggests the possibility of establishing new cell lines from primary tumors at different stages, by using the applications of gene expression profiling and improved methods for culturing primary cells.

Nowadays, the BCSCs hypothesis has attracted a lot of attention due to its unique properties and possible prognostic applications. Thus, it is essential to ask whether breast cancer cell lines contain any BCSCs subpopulations. So far, some laboratories have reported that they have successfully identified subsets of cells having distinct tumor initiating phenotypes within various cell lines[162,241-243]. For instance, MCF-7 is enriched in the subpopulation of cells able to form mammospheres, exhibiting the surface marker $CD44^+/CD24^-$ profile, which is 1000 times more capable of initiating tumors in mice compared to the parental cells [244].

Kuperwasser and colleagues performed *in vivo* limiting dilution transplantation to test the tumorigenic capability of $CD44^+/CD24^-$ subpopulations from breast cancer cell lines. They noticed these markers did not correlate with tumor initiation and instead indicated a basal-like subtype. However, when incorporating the ESA^+ fraction together with the CD44+/CD24- cells, tumorigenic capability of the subset increased [245].

Accumulating evidence demonstrates that the $CD44^+/CD24^-/ESA^+$ phenotype varies within both cell lines and breast cancers, suggesting that the utility of surface markers is not as broadly indicative as expected for tumor initiating capacity [141,246,247]. Besides, the correlation of surface marker phenotypes to clinical outcomes has not yet been established [141,246,248]. Thus, more systematic functional studies are needed. In the meantime, utilizing different cell lines to represent breast cancer subtypes is also necessary to provide solid conclusions about the CSCs compartment in each cell line, and whether they display identical surface markers for further identification. Despite their limitations, there are several studies suggesting that certain breast cancer cell lines can be used to investigate the differences of cellular and molecular components between tumorigenic and non-tumorigenic subpopulations [162,244,249].

1.5.2 *In vivo* models

The orthotropic transplantation of breast cancer cell lines into xenografts allows the investigation of tumor growth *in vivo*, by which the complex tumor stromal cell interaction and microenvironments that facilitate tumor initiation and progression can be taken into consideration. Using xenografts has become a gold standard model to study the facts of breast cancer biology, including the role of tumor extracellular matrix interactions, angiogenesis, inflammation, the genetic alterations that contribute to tumor formation and growth, and even the transformations that lead to stepwise metastasis [250]. However, there are still some aspects that need to be carefully considered when using xenograft models.

A crucial limitation of this model is that using cell lines to reform tumors is unable to represent all five common subtypes of breast cancer and their intrinsic heterogeneity as observed *in vivo*. Technical aspects that can influence the utility of xenografts should be mentioned as well. First of all, xenografts to model breast cancer have always been established in immunodeficient mice without intact

immune systems, which might profoundly affect tumor development and progression [251]. Many studies have shown that immune system interactions in the early stages of tumor formation and later in metastasis play an important role in breast cancer [252,253]. In addition, the tumor cells are frequently placed into the flank of mice by subcutaneous injection and it is not difficult to conclude that this microenvironment is very different from the real intratumoral microenvironment and thus alters the growth potential of engrafted cells. Orthotropic transplantation into the mammary gland might be more favorable, yet there are huge differences between human and mouse mammary stroma. Alternatively, introduction of tumor cells into the mammary fat pad is also widely used, although the epithelial and stromal component interactions cannot be represented in the mouse mammary fat pad [254]. Using mouse models to investigate metastasis also has limitations. In mice, metastatic cells usually fail to grow at other distant sites but prefer to colonize the lungs, which does not represent the situation in humans, where metastasis most often occurs in the lymph nodes, liver, bone and brain [255].

However, modeling breast cancer in xenografts is still relevant to human cancer prognosis. In a recent and elegant study, the breast cancer cell line MDA-MB-231 was used by intravenous injection to select for metastatic variants to the lungs. Molecular profiling was performed to generate a metastatic gene signature from the lung metastatic variants. Interestingly, this defined gene signature significantly correlated with specifically distinguished patients who relapsed with lung metastases and decreased metastasis-free survival [256]. Other studies have also identified gene signatures from xenografts that are associated with distinct outcomes [257,258]. These inspiring studies demonstrated the utilities of xenograft models and their relevance to breast cancer in humans.

In recent years, the development of patient-derived xenograft (PDX) models has provided a unique tool to investigate breast cancer biology in a more clinically oriented context. These xenografts can be generated from a wide range of human cancers and able to preserve inter- and intra- tumor heterogeneity [259]. Usually, fragments of human tumors are directly implanted into immunocompromised mice. Therefore, the regrown tumors often retain the histological characteristics of the parental tumors. Numerous studies have shown PDX models even preserve mutation profiles as well as maintain similar response patterns to different therapies[259-262]. Some PDX models are also useful for evaluation of post-therapeutic tumor characteristics. PDX models also allow for the interactions between tumor cells and stromal compartments that are structured in human cancers [263]. However, those studies were only able to describe results from a limited amount of samples and subtypes because the transplantation efficiencies are reported to be low.

1.5.3 *Ex vivo* models

Representing breast cancer diversity is an issue that should be considered when choosing model systems. The heterogeneity usually cannot be easily or accurately replicated in routine cancer cell lines or xenograft models as previously discussed.

The former models lack tumor complexity, while the latter models often suffer from inadequate human mammary gland and physiological environments. Alternatively, organotypic tissue slice culture offers an attractive *ex vivo* model, as not only can it preserve inter- and intra- tumor heterogeneity, but also allows for evaluation of drug response directly in the culture and subsequent downstream analysis [264-266]. In breast cancer, the evaluations of this humanized model have been proven to reflect the morphological and histopathological properties of the parental tumors [267]. Careful technique allows for culturing tissue slices for up to 7 days, although downstream processing is somewhat challenging [268]. Some studies have assessed the synergistic effects of compounds with potential anti-cancer effects with this model, indicating that organotypic culture can respond to different magnitudes of treatment. These findings further reflect the importance of taking tumor heterogeneity into consideration when modeling breast cancer [269].

Overall, the *ex vivo* model has advantages in conducting a large number of assays that can capture many aspects of tumor diversity, including cell matrix components, inter- and extracellular interactions, and the most important metabolic capacities. It also provides the possibility to study different aspects of cancers. It could also contribute to a decrease in the number of experimental animals consumed in cancer research. More studies should be performed using this model.

2 AIMS OF THE THESIS

The aim of this thesis is to further explore the properties of breast cancer stem cells and to better characterize this subpopulation. We also aim to highlight their importance in association with clinical outcomes, and therefore they may be helpful in developing targeted therapies for future clinical use. Finally, we aim to establish a better system to preserve and expand primary cultures from breast cancer specimens in order to benefit future research.

Paper I: to identify the role of ERβ as a mediator of estrogen action and the possibility of using it as a novel target for endocrine therapy in BCSCs.

Paper II: to investigate the reversibility of BCSCs dedifferentiation from bulk tumor cells and to explore whether putative BCSCs display tumorigenicity as a certain genotype within tumors.

Paper III: to explore the effect of tamoxifen on the patient-derived BCSCs, and to understand the potential mechanism of BCSCs-induced endocrine resistance in breast cancer.

Paper IV: to establish a simple and standardized method to generate material from small breast tumors without risking the histopathological examination, and to isolate and expand cancer stem cells from the majority of tumors collected for research purposes, biobanking, and next-generation sequencing.

3 MATERIALS AND METHODS

The unique and major methods employed in this thesis to generate results are described below.

3.1 *IN VIVO* MODELS

3.1.1 Uppsala cohort

The Uppsala breast cancer cohort was first described in 2005. It consists of 315 breast cancer patients who had been diagnosed from 1987 to 1989 in Uppsala County in Sweden. It included 65% of all breast cancer patients at that time in Uppsala. The clinical reports and histopathological characteristics were retrieved from the patients' records. Long-term patient follow-up performed by examining the survival status and the cause of death has been conducted several times using the registries. Global gene expression analysis was performed on 260 of the patients with Affymetrix microarray chips. An intrinsic subtype analysis was performed to classify the patients. Tissue Microarrays (TMAs) have also been constructed from the original formalin fixed paraffin embedded tumor tissues, which were used to test for ERβ status and cancer stem cell surface markers in paper I.

3.1.2 Mouse tumor model

The experimental tumor models in laboratory animals included the orthotropic model, the transplant model, and the spontaneous model. The advantages of using mouse models to study human cancer biology have been discussed in the Introduction. In our projects, cell pellets from different breast cancer cell lines were orthotropically transplanted into the 4^{th} pair of mammary fat pads for tumor initiation and out-growth. Treatments were administrated by subcutanenous injection according to the experimental purposes. When tumor growth was larger than 3 cm in diameter, the mice were euthanatized using a CO_2 chamber. The extracted tumors were collected for subsequential validation.

3.1.3 Mouse surgical applications

According to the object of each animal experiment, mice were subjected to certain surgical procedures to create physiological relevant models and to mimic the cancer environment in humans. In our projects, when studying ERα agonizing and antagonizing molecules, or specifically focusing on ERβ funtions, the mice were ovariectomized to diminish the interference from endogenous estrogens. In other cases, the mammary gland was removed from the mice at a young age before transplanting cancer cells into the cleared mammary fat pad. This was done in order to prevent the potential mammary ductal tree's extension throughout the mammary fat pad and therefore disturb exotic cell growth.

3.2 *IN VITRO* MODELS

3.2.1 Immunofluorescent staining

Immunofluorescence was intensively utilized in our projects to identify the properties of mammospheres from clinical material. This microscope-based technique enabled the characterization of cancer stem cells based on their surface marker status such as CD44, CD24 and ALDH1. In our experimental settings, limited access to clinical breast cancer specimens increased the difficulties in handling such delicate material. Therefore, we set up a panel of stable and repeatable immuno-fluorescent staining techniques to study cancer stem cells and their functions when responding to various conditions. Small amounts of spheres were cytospun onto slides and incubated with the antibodies of interest. After signal amplification by secondary antibodies, we were able to observe protein expression levels and interactions between proteins under the microscope. We believe this is a very sensitive technique in terms of its implementation in cancer stem cell research. It is worth noting that choosing validated primary antibodies is essential and critical for generating reliable results.

3.2.2 Mammosphere formation assay

The mammosphere formation assays have been widely used for their ability to identify cancer stem cells based on their self-renewal, differentiating and slow proliferating capacity at the single cell level *in vitro*. We mainly used the sphere formation assay to detect the potential effect of molecules in targeting identified BCSCs when stimulating or inhibiting different subcellular compartments in the cells. In general, spheres derived from either patient material or cell lines were trypsinized into single cells and re-seeded into culture plates, then they were incubated with a series of treatments over a certain time, to observe the changes in the newly formed sphere number.

3.3 *EX VIVO* MODEL AND SUPERFICIAL SCRAPING TECHNIQUE

3.3.1 Primary breast cancer cell culture

The advantages of using primary cultures derived from breast cancer specimens over traditional cell lines have been extensively discussed in the introduction. The major obstacle in long-term culturing of primary cells is how to maintain the phenotype and limit genomic changes compared to parental tumors once extracted outside physiological conditions. We therefore mainly used first-generation mammospheres purified from patient material in all of the projects to limit genetic and phenotypic changes introduced during the culture. To select target cancer stem cell populations, we used restricted medium as reported in the thesis paper IV, to which normal serum was not added. By doing so, cells were not able to attach and undergo differentiation but instead displayed self-renewal and a slow proliferating capacity.

3.3.2 Biobanking superficial scrapings from breast tumors

In this thesis, the establishment of the superficial scraping technique is a unique technique that is important for future research and clinical purposes. A sterilized scalpel was scraped against the surface of the resected breast tumor several times by a trained pathologist. The cell scrapings were immediately transferred either into a cryopreservation vial for biobanking or into selective medium for culturing (Figure 3). This method was validated to provide sufficient material for later gene expression profiling, next generation sequencing analysis and *ex vivo* cell models.

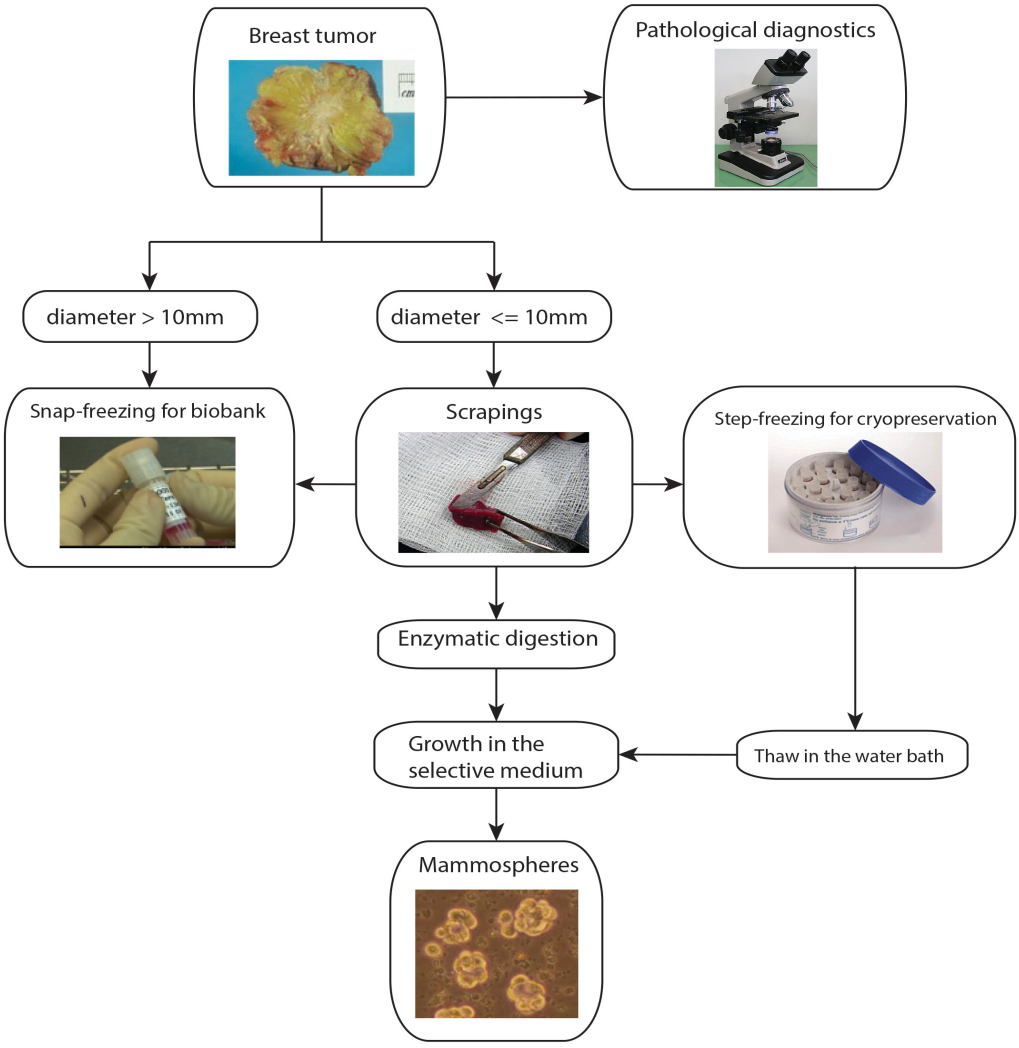

Figure 3. The flow chart illustrates the process and procedures devised to evaluate the feasibility of biobanking the superficial scrapings and as a resource of cancer stem-like cells.

4 RESULTS AND DISCUSSION

4.1 PAPER I

Estrogen receptor β governs proliferation of breast cancer stem cells and can be targeted by endocrine therapy

Breast cancer stem cells (BCSCs) are highly tumorigenic cells expressing classical embryonic stem cell genes such as *NANOG*, *SOX2* and *OCT4*, with a capacity to self-renew and further differentiate into a heterogeneous tumor mass. These cells are defined by their potential in growing as non-adherent spheres *in vitro,* together with their distinct cell-surface antigenic profile $CD44^{+}/CD24^{-/low}$ as well as high ALDH1 expression. It has been suggested that BCSCs are responsible for therapeutic resistance, governing the metastatic process and tumor cell dormancy in the late stages of disease.

We first evaluated ERβ protein expression with molecular subtypes in a cohort of 187 breast cancer patients with available gene expression-based subclass categorization, and concluded ERβ expression was evenly distributed across all breast cancers. Dual immunohistochemical staining with ERβ and the BCSCs surface marker CD44 was performed on the tissue microarray from the same cohort. Observed ERβ expression highly overlapped with CD44 expression (71%). After successfully isolated BCSCs (mammospheres) from breast cancer biopsies, we performed immunofluorescent staining on another 45 patients, and confirmed that the vast majority of mammospheres were $ERα^{-}$ $ERβ^{+}$, $CD44^{+}/CD24^{-}$, high ALDH1 (>95%) and positive for PKH26, again showing that ERβ expression in BCSCs was independent of both tumor subtypes and ERα status. A similar result was also detected from mammary stem cells (MSCs).

To further explore the mechanistic role of ERβ in BCSCs maintenance, we employed the MCF7 (luminal-like) and MDA-MB-231 (basal-like) cell lines, and generated spheres from these as surrogates for patient-derived BCSCs. We could confirm these spheres were phenotypically similar to those of clinical *ex vivo* material by immunofluorescent staining. Reverse transcriptase-polymerase chain reactions (RT-PCR) showed that the cell line spheres were expressing high levels of ERβ along with the embryonic pluripotency genes *SOX2*, *NANOG*, *OCT4*.

To assess the importance of ERβ for maintenance of the stem cell state, we first knocked out ERβ in the MCF7 and MDA-MB-231 spheres. Suppression of ERβ resulted in a significant reduction in mammosphere formation. In contrast, overexpression of exogenous ERβ increased the sphere-forming capacity in each generation of cell culturing. Forced-differentiation of clinical BCSCs reduced ERβ and ALDH1 expression. We also performed proliferation and sphere formation assays. In cell line models, we found the ERβ-selective agonist diarylpropionitrile (DPN) induced cell proliferation only in spheres but not in adherent cultures. This was confirmed in clinical BCSCs as well. More interestingly, treatment with

tamoxifen, fulvestrant, or the ERα-selective agonist propylpyrazoletrisphenol (PPT) did not cause any significant change in mammosphere formation in clinical BCSCs. When the same treatments were applied to MSCs, stimulation of ERβ by DPN caused a dramatic reduction in mammosphere formation.

To further explore ERβ function in BCSCs, we performed whole-transcriptome analysis of adherent MCF7 and mammospheres (MCF7S) incubated with either a vehicle control or the agonist DPN. Transcriptomal changes after DPN treatment in MCF7 and MCF7S revealed separate and distinct gene expression signatures. Gene-set and pathway enrichment analysis found that the "HIF1α transcription factor network" and "genes involved in glucose metabolism" were the most significantly enriched pathways. Afterwards, clinical associations were performed using the signature generated from DPN-stimulated MCF7S. We found high MCF7S DPN signature scores were statistically significantly associated with poor distant metastasis-free survival in three independent breast cancer cohorts.

To assess the effect on tumor-initiating capacity of ERβ *in vivo*, we transplanted MCF7 and MDA-MB-231 cells in the form of spheres or adherent cultures into immune-deficient xenografts according to the experimental purposes. We observed that when ERβ was suppressed either by an inhibitor 4-[2-Phenyl-5,7-*bis*(trifluoromethyl)pyrazolo[1,5-*a*]pyrimidin-3-yl]phenol (PHTPP) or by knock-out, the tumor volume was dramatically reduced whereas stimulation of ERβ by DPN gave rise to larger tumor growths. In compliance with previous results, when tumors were extracted and immunostained with different markers, we found PHTPP could either reduce the number of mitotic cells or decrease ALDH1 positivity in the cells stimulated by E_2. We also assessed the tumor suppressive role of PHTPP on clinical BCSCs. As expected, PHTPP completely abolished the stimulatory effect of E_2 on sphere formation.

Finally, we investigated whether a combination of tamoxifen and PHTPP would be more efficient in blocking tumor growth. The results indicated that tamoxifen alone was not able to eliminate the tumor completely. Combining tamoxifen with PHTPP caused a gradual decrease in tumor size in response to increasing PHTPP dosage.

In conclusion, ERβ is expressed evenly within all breast cancer subtypes and the expression of ERα is completely negative in cancer stem cells at the protein level, whereas it appears to be highly expressed in the majority of differentiated tumor cells. ERβ protein is expressed in the majority of cancer stem cells, and its expression declines as the cancer stem cells differentiate. Therefore, we hypothesized a shift from a high to a low ERα/ERβ-ratio correlates with the tumor progression process. Our data from the proliferation and sphere formation assay illustrate ERβ as the predominant estrogen receptor that is essential for both normal and cancer stem cell phenotype maintenance. *In vivo* data suggest that ERβ inhibitors together with conventional endocrine therapy would be more efficient in targeting both the more differentiated (mainly ERα+/ERβ+) cells as well as the stem-cell-like, poorly differentiated cells (ERα-/ERβ+). We thereby identify ERβ as

a mediator of estrogen action and a novel target for endocrine therapy in BCSCs.

4.2 PAPER II

Sequencing of breast cancer stem cell populations indicates a dynamic conversion between differentiation states in vivo

Currently, two alternative and contradictive explanations have been proposed to describe the tumor progression process governed by CSCs in breast cancer (Figure 4). In the first classical cancer stem cell model, a highly differentiated bulk tumor is generated as a consequence of proliferation and irreversible conversion from cancer stem cells. Therefore, an accumulation of unique mutations with low allelic frequencies should be observed in the rest of tumor. In the plasticity model, the cancer stem cell phenotype and the stochastic state in a bulk tumor maintain an equilibrium and a reversible transition through de-differentiated modifications. In this model, mutations and allelic frequencies should display high similarities between these two compartments.

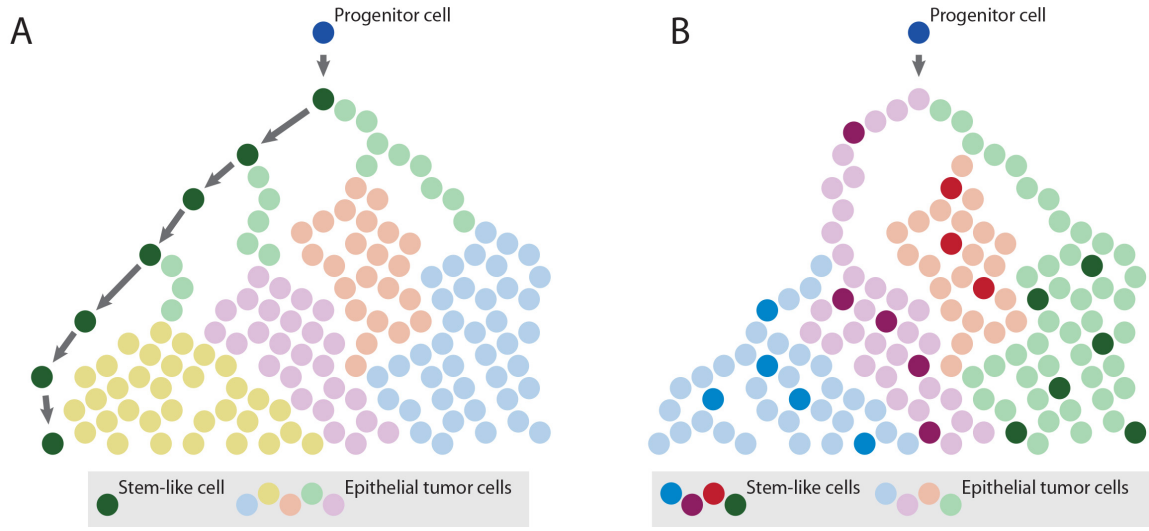

Figure 4. Two main hypothesis of tumor progression by BCSCs. Stem-like cells and differentiated epithelial tumor cells are labeled as different colors.
(A) BCSCs irreversibly convert into other cells and undergo asymmetrical division to produce a hierarchical bulk tumor. (B) Plasticity and dynamic transition occasionally occur between BCSCs and differentiated tumor cells within a bulk tumor.

To investigate the hypotheses, we compared the spectra of mutational changes between breast cancer stem cell populations and the rest of the tumor cells from the same biopsy. The compartment of cancer stem cells were identified either by culture as mammospheres *in vitro* and characterized by a serious of experiments, or by direct cell isolation using fluorescent-activated cell sorting (FACS) based on their surface marker profile $CD44^{+}/CD24^{-}$, or high ALDH1. Mammospheres and biopsies were isolated from 10 patients and subjected to whole exome sequencing.

Massive bioinformatics have been performed to detect the somatic mutational patterns within these two compartments from each patient. The investigation of the extent of shared mutations between BCSCs and primary bulk tumor found these to highly overlap. On average, 83% of the mutations were shared. The difference between those shared mutations and the total number of mutations in bulk tumor was not statistically significant. Uni- and multivariate analysis after adjustment for tumor characteristics still indicated no significant differences. The mutated allele frequency of the shared mutations was also evaluated, and it was shown to be similar between the BCSCs and bulk tumor as well. The same procedure was repeated on the mutations that were unique to each compartment, and the result showed the frequencies of unique mutations were significantly lower than the shared mutations.

To validate the detected somatic mutations from exome sequencing analysis, ultra-deep amplicon sequencing across selected 14 mutation sites (based on the mutational pattern) was performed on three patients. Using this method, only one mutation at a low allele frequency (5%) could be defined as BCSCs-unique and the rest of the mutations were either shared or found to be false positive from the first round of analysis.

Because there is some controversy about using mammospheres to define BCSCs populations, we also isolated two other subpopulations of BCSCs carrying the $CD44^{+}/CD24^{-}$ or high ALDH1 profile by FACS sorting. After exome sequencing on these two subsets compared to their non-CSCs component $CD44^{-}$ or low ALDH1, respectively, the results again revealed the similarity of shared mutational patterns between BCSCs and non-CSCs populations. Our data showed the somatic mutations in the BCSCs state and in the differentiated cell state were highly similar, as were the spreading allele frequencies over the spectrum. This finding was consistent across different methods to identify BCSCs populations.

Our results support the plasticity model of BCSCs existence in the bulk tumor. First, we observe a high degree of shared mutations between BCSCs and the differentiated tumor cells, implying the occurrence of transition and inter-conversion within these two cellular phenotypes. Hence, the dynamic cell state model is a better fit to the data. The large spread in allele frequencies seen in the two components also provides strong evidence that as early mutations occur in the rapidly dividing differentiated epithelial cells, the propagated offspring cells are occasionally reverting to stem-cell-like cancer cells, which harbor the same mutations. Since these early events are represented as high allele frequencies, it directly points out the fact that mutations accumulated in early tumorigenesis are able to be transmitted across all of the cell states during tumor progression; therefore, BCSCs can carry similar frequencies to those in the non-tumorigenic cells. Although it has been noted that allele frequencies alone are inadequate to classify different subclones distinctively. In conclusion, we assume BCSCs as well as differentiated tumor cells exist in parallel in a bulk tumor rather than being distinct subsets of cells on the apex of a tumor progression hierarchy.

4.3 PAPER III

mTOR inhibitors counteract tamoxifen-induced activation of breast cancer stem cells

In paper I, we noted that although tamoxifen could efficiently inhibit adherent cells from proliferating, such as the MCF7 cell line, this effect appeared to be insufficient to reduce sphere formation from patient-derived BCSCs. We decided to investigate the mechanism behind this intrinsic resistance to tamoxifen in mammosphere. Interestingly, we and others have proved that BCSCs are low in ERα and they appear not to be a distinct cancer cell lineage but are repeatedly created through dedifferentiation of bulk tumor cells. The comprehensive mechanism of tamoxifen resistance in ERα positive breast cancer has not yet been fully discovered, but the leading hypothesis involves cross-reactivity with other growth factor signaling pathways or through imbalanced PI3K/AKT/mTOR signaling. These two possibilities have been suggested to be causes for acquired endocrine resistance in tumors retaining or gradually losing ERα. We were interested to understand whether the observed resistance in BCSCs had a similar mechanism, and whether the mechanism in *ex vivo* models could contribute to explaining the intrinsic tamoxifen resistance observed in patients with ER-positive tumors.

We isolated mammospheres from several patients and immuno-stained with CD44, CD24, ALDH1, EpCAM, and other markers to characterize the BCSCs phenotype. We consistently observed that mammospheres, although a minor component in the bulk tumor, were enriched in the subset of cells carrying all of the required BCSCs properties. This finding was also observed in the cell-line-generated spheres.

Patient-derived and cell-line models were treated with tamoxifen. Tamoxifen failed to prevent sphere formation in both patient-derived BCSCs and cell line BCSCs, in contrast to its antagonizing effect on the adherent cultures. Mammospheres were purified from another seven breast cancer patients, and they were treated with tamoxifen or with the vehicle control. After performing whole transcriptome analysis, it was found that treatment with tamoxifen triggered a distinct gene expression pattern compared to the control.

Gene-set enrichment analyses were performed to explore the biological relevance of this differential expression pattern. The most significant pathways activated by tamoxifen in BCSCs were ribosome synthesis and mRNA translation. These indicated a highly activated mTOR-signaling pathway enhanced by tamoxifen. We therefore selected well-characterized downstream effectors of mTOR to validate the hypothesis. In western blotting results, two patient-derived BCSCs cultures showed significant induction of phosphorylated S6RP by tamoxifen whereas in MCF7 and T47D spheres, phosphorylated 4E-BP1 was dramatically induced by tamoxifen. From immunofluorescent staining, phosphorylated S6RP induction can be detected in all of the cell cultures, although the self-stimulation was higher in the cell lines and the level of induction varied among patients. Notably, all of these mTOR effectors, which were highly expressed in BCSCs after tamoxifen treatment,

can be attenuate by a series of mTOR inhibitors. Again, when mTOR inhibitors were employed, either alone or in combination with tamoxifen, they could effectively reduce mammosphere formation.

In conclusion, our data support the idea that tamoxifen may work as a partial agonist on BCSCs by activating this population and further driving relapsed tumor to become tamoxifen resistant. Based on the tamoxifen-induced transcriptome in BCSCs and the proposed central role of mTOR governing endocrine resistance, we hypothesize that tamoxifen stimulates the mTOR pathway; thereby tamoxifen would increase the translational process for maintenance and viability of BCSCs. Of note, the mTOR signaling pathway is already active in BCSCs but is further stimulated by tamoxifen treatment, and this effect is independent of ERα status and can also be observed in triple-negative patients. While antagonizing the mTOR activation by mTOR inhibitors, we noticed constitutive AKT activation that was not fully eliminated by rapa-analogs. It should be taken into consideration that high levels of p-AKT might reflect a mechanism of rapamycin resistance in paradoxical compensation initiated by mTOR inhibition. This is supported by previous studies reporting the problem with mTOR inhibition is the risk of blocking the negative feedback loop effect of insulin-like growth factor-1 receptor (IGF-1R) on AKT/PI3K signaling by increased AKT phosphorylation, and further abrogating the therapeutic effect. We report that a dual PI3K/mTOR kinase inhibitor, PF-04691502, exhibited robust antitumor potent and exerts a preferential effect in targeting the BCSCs component. We suggest that a combined treatment of tamoxifen and rapalogs is a way to eradicate the CSCs population more efficiently in parallel with the bulk tumor.

4.4 PAPER IV

Superficial scrapings from breast tumors is a source for biobanking and research purposes

Nowadays, rapid evolving technologies such as next generation sequencing (NGS) pave the way forward to better diagnosis and therapy in breast cancer. Accordingly, clinical pathology laboratories would also expect to update their tissue-handling pipelines to adapt to new demands for both scientific research and clinical uses. This requires guaranteed material quality, such as stable mRNA, DNA and protein, without compromising pathological diagnosis and the materials need to be representative compared to the original breast cancer specimen. Fresh-frozen tissue is the ideal, and is the only way to preserve mRNA, DNA and protein efficiently for subsequent analysis. However, current guidelines for biobanking breast cancers requires tumor specimens to be at least 1 cm in diameter, and nearly 25% of breast tumors are smaller than 1 cm. Since mammography screening dramatically increases the chances of discovering tumors at early stages, the percentage of small breast cancers will be expected to increase in the future. We have established a simple and standard method, using superficial scrapings, to biobank high-quality samples from small tumors without disrupting histopathological examination.

Since taking biopsies from breast cancers might introduce dilution and contamination by stromal cells or other cell types, we first assessed the cell component from superficial scraping in several patients. Cytological smear material was examined by a cytologist and revealed that the majority (>95%) of the scraping was composed of cancer cells. We also cultured the scrapings in selective cancer stem cell media and found mammosphere formation. With the previous knowledge of the mammosphere as a cancer stem cell enriched phenotype, we characterized those cells through a series of experiments and defined them as $CD44^+/CD24^-$, high ALDH1, $EpCAM^+$ and $PKH26^+$. We also generated adherent cancer cells by plating scrapings in the differentiating cell medium.

To assess the sample quality, scrapings from tumors were directly transferred into cryopreservative tubes. The tubes were immediately put into liquid nitrogen and stored at -80ºC. After 1 week, samples were thawed and DNA or mRNA was isolated. We also isolated mRNA from paired bulk tumor pieces as a comparison to assess mRNA recovery efficiency from the scrapings. After analysis on the basis of microchip gel electrophoresis and quantification by measuring the total absorbance, we observed distinct ribosomal peaks and a decent RNA integrity number (RIN) value from scrapings mRNA, indicating no severe degradation occurred during the biobanking procedure; also, the mRNA yields from scrapings were of acceptable purity, thereby enabling usage of scraping material for gene expression assays.

To investigate whether the superficial scrapings can truly represent the gene expression pattern of the original breast cancers, we performed RT-PCR on both scrapings and corresponding breast cancer biopsies. Results showed scrapings shared similar expression patterns of *ERα* and *PR*, but with a varied expression level compared to the parental tumor. They also expressed much lower levels of fibroblastic markers (*PDGFRβ*, *α-SMA*) and higher levels of the epithelial marker *EpCAM*. This reflected the enriched fraction of cancer cells in the superficial scrapings.

Scraping DNA quality assessment was also performed. The results indicated proper DNA yields, and sufficient purity and integrity for next generation sequencing after low temperature storage. Additionally, we investigated the usage of scraping material for epigenetic studies. Scraping DNA extracted from two ERα positive patients was used to analyze the ERα methylation status using the pyrosequencing technique. Analysis indicated the scrapings retained the key methylation signatures, which correlated to the gene expression of the bulk tumor itself.

The conducted validations on the scrapings from dissected tumor surface strongly suggest they could be successfully used for investigations at the DNA and mRNA level. Apart from their use for gene expression and next generation sequencing, these materials are also valuable for cancer stem cell research and methylation studies, since accumulating reports show the importance of considering cancer

stem cells for potential relapse, metastasis and treatment resistance. Studies at the epigenetic level appear to be promising methods for therapeutic response prediction. However, we could not use this material from small tumors for current proteomics investigations since the input amount is required to be in the 20-50 µg ranges. Currently, at the Department of Pathology in Karolinska University Hospital, the routine biobank procedure through standard biopsies taken from fresh tumor tissue includes approximately 60% of all cancers. By using this simple and robust technique using scrapings, we could also collect fresh cells from small tumors and further increase the biobank inclusion of up to 85% of all resected tumors. In summary, we conclude superficial scrapings are as an effective source for both biobanking and research purposes.

5 FUTURE PERSPECTIVES

Breast cancer stem cells - myths or facts?

The concept of breast cancer stem cells has been established over decades. However, none of the reports ever precisely defined the real cancer stem cells in a bulk tumor. All of the conclusions up-to-date are merely based on cell surface antigen profiles or certain patterns of protein expression status to isolate a subset of cellular components within solid tumors. Their existence can only be indirectly proved as only a few isolated cells subjected to xenograft ever give rise to an integrated tumor.

As discussed previously, several groups have evaluated genotypes and biological functions of BCSCs purified using different isolating methods or different subtypes of breast cancer. There are several key findings: 1. BCSCs isolated by different methods display different lineages during cancer progression; 2. BCSCs isolated from different subtypes of tumors harbor inter-tumor heterogeneity; 3. Not all of the BCSCs generated from a certain subtype of breast cancer can successfully reform a parental-tumor-like secondary tumor in the xenografts. Therefore, BCSCs seem to be a distinct small group of cells only within an individual tumor. In addition, the theory of BCSCs fails to explain how breast cancer initiates and progresses to develop inter- and intra- tumor heterogeneity during the later stages.

This heterogeneity is an interesting issue in breast cancer evolution, once revealed, at least it can serve as a more precise prognosticator to define patients under high risk of breast cancer occurrence at certain ages. We and others have reported that current BCSCs are only a phenotype that occasionally dedifferentiates and re-forms from other differentiated epithelial cancer cells. Hence, as currently defined, BCSCs are insufficient to fully explain breast cancer initiation and cannot be utilized for predicting breast cancer occurrence.

However, in my personal opinion, the importance of recognizing current BCSCs isolated by different methods matters more in how they influence therapeutic outcomes. First, it has to be emphasized here that the present conception of BCSCs should be better understood as a niche where several cell components coordinating together act on similar stem-cell-like behaviors. *In vitro* studies showed BCSCs characterized from both breast cancer patient material and different cell lines all exhibit unique responses to conventional therapies (tamoxifen, chemo-, radio- therapy) compare to other cells from the same source. However, they are able to form an integrated tumor display with molecular profiling similar to the original tumor when subject to xenografts, although the tumor take-out ratio is quite low.

Taken into consideration all of facts discussed above, it is possible to conclude the following: 1. Currently-defined BCSCs perform their therapeutic resistance function not as consequence of a single cell or real cancer stem cells, it through the cooperation with other cells in the same niche and further facilitated by surrounding tumor cells. 2. Long-term conventional therapies can increase the occasions that force cancer epithelial cell dedifferentiation to form more BCSCs. 3. Induced an increased number of BCSCs or already existed BCSCs surviving from conventional therapies can lead to further resistance to various therapies. 4. As long as they survive, BCSCs can reconstruct a

second tumor in the form of local relapse or distant metastasis, although the chance of this happening might be low. Therefore, studies focusing on BCSCs peculiar biological features and functions are highly encouraged. Administrating treatments to target BCSCs specifically seems to be inferior to a combination of treatments targeting both compartments. A novel therapeutic strategy should be established for treating differentiated cancer cells in parallel with eliminating BCSCs more efficiently.

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All the PIs in 3rd floor

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Team Johan:

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Maria Masucci's group

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